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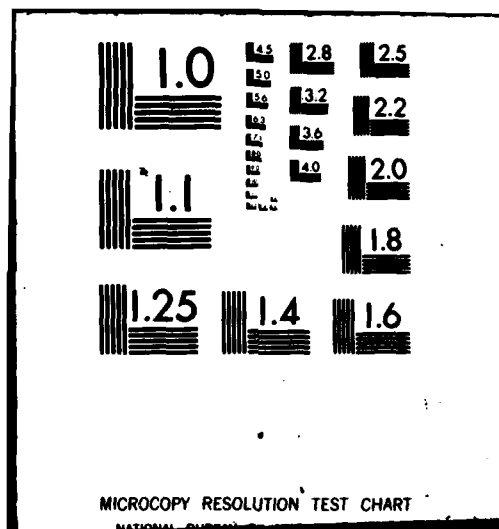   JUN 82 P MIKESELL, B B KNUDSON

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PLASMIDS OF LEGIONELLA SPECIES

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Plasmids are extrachromosomal genetic elements of bacteria that are not essential for survival of the host under most circumstances but often carry genes that allow the host to compete more successfully in adverse environments. Plasmids have been shown to carry genes which code for toxins, such as botulinum, diphtheria, streptococcus erythrogenic, and the enterotoxin of Escherichia coli (1). In addition to carrying genes responsible for metabolism, resistance to metals and fertility factors, plasmids also encode for drug resistance factors. These latter genetic elements are an important consideration in the treatment of infectious diseases, particularly in treating pathogens responsible for nosocomial infections. The sudden outbreak of a highly infectious disease caused by a multiply drug-resistant organism could severely impact on the planning and progress of a given military operation. Areas of high troop concentrations, such as basic training units, are particularly susceptible to the rapid spread of an infectious disease.

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There are approximately 800,000 cases of pneumonia in the United States each year for which no known viral or bacterial agent can be identified (2). An outbreak of pneumonia occurred in 1976 at the Bellevue-Stratford Hotel in Philadelphia during an American Legion convention (3). The etiological agent was identified as a fastidious, aerobic, gram-negative bacterium and given the name Legionella pneumophila. The number of reported cases of legionellosis is rising rapidly and it is probable that a significant number of here-to-fore undiagnosed cases of pneumonia can be attributed to this novel pathogen.

The possible involvement of plasmids in legionellosis was suggested by reports of cytotoxin (4) and β-lactamase production (5) by L. pneumophila and by reports of virulent to avirulent conversion of the organism through serial passage on artificial media. Another strong indicator that plasmids may be involved in the pathogenesis of legionellosis was suggested by the very narrow spectrum of antibiotics effective in the treatment of the disease. We were interested in examining strains of Legionella for

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the possible presence of plasmid DNA and determining what if any role
these elements play in the pathogenesis of the disease.

MATERIALS AND METHODS

In the initial phase of our study, 16 strains from the six serogroups
of L. pneumophila were examined for the presence of extrachromosomal DNA
by a modified cleared lysate technique. Bacteria were harvested by scrap-
ing 10 charcoal yeast extract agar plates, which exhibited confluent
growth, using a bent glass rod and 3 ml per plate of saline containing 0.1%
tryptose, pH 7.0. Each strain was also harvested from 400 ml of yeast ex-
tract broth culture at ca. 2×10^9 cells/ml by centrifugation at 10,000 x g
for 10 min at 4°C. The pellet was resuspended in 25 ml of 25% sucrose so-
lution made up in 0.05 M tris (hydroxymethyl)aminomethane (Tris), pH 8.0,
at room temperature. Spheroplasts were generated by the addition of 3.0 ml
of freshly prepared lysozyme solution (10 mg/ml in 0.25 M Tris, pH 8.0).
The suspension was mixed and placed on ice for 5 min. A 3.0-ml portion of
0.25 M ethylenediaminetetraacetate (EDTA), pH 8.0, was then added, and the
mixture was chilled in an ice bath for an additional 5 min with occasional
swirling. Lysis was brought about by adding 30 ml of 2.0% Triton X-100
made up in 0.025 M EDTA and 0.05 M Tris, pH 8.0. The final mixture was
incubated at 37°C for 15 min and gently swirled until clearing occurred.
The viscous lysate was centrifuged at 4°C for 30 min at 48,000 x g to sedi-
ment most of the chromosomal DNA and cellular debris. The plasmid DNA re-
mained in the supernatant; it is referred to as cleared lysate.

The supercoiled plasmid DNA in the cleared lysate was further purified
by CsCl-ethidium bromide (EtBr)-density gradient centrifugation (6). One
gram of solid CsCl and 0.1 ml of EtBr solution (5 mg of EtBr per ml in TE
buffer; 10 mM Tris, 1 mM EDTA, pH 8.0) was added per milliliter of lysate.
The EtBr-DNA complex was protected from light to prevent light-activated
dye nicking of the covalently closed circular (CCC) DNA. The bands were
located by using long-wave ultraviolet illumination and were removed
through the side of the tube with an 18-gauge needle and syringe. The
dense satellite bands containing the CCC plasmid DNA were then pooled, and
the EtBr was extracted three times with an equal volume of isopropanol (pre-
saturated with water and CsCl). The samples were then dialyzed against
three 1-liter changes of 15 mM NaCl and 1.5 mM sodium citrate and stored at
4°C.

Samples of 40 to 100 µl of plasmid preparation were mixed with 20 µl
of tracking dye solution (0.7% bromophenol blue, 7% sodium dodecyl sulfate,
and 16.5% glycerol in water) and subjected to electrophoresis, similar to
the method described by Meyers et al. (7), DNA was electrophoresed in both
0.7 and 0.8% agarose with Tris-borate buffer (89 mM boric acid), pH 8.3,
on a vertical slab gel. Electrophoresis was carried out at 120 V and 20°C
for 2.5 h, or until the dye reached gel bottom. For comparative purposes,
samples were also electrophoresed at 30 V for 16 h. The gels were stained
for 30 min in an aqueous EtBr solution (0.5 µg/ml) and then washed in

water for 20 min. Plasmid DNA bands were visualized using ultraviolet light.

The second phase of our study was to examine another group of bacteria which were classified as Legionella-like organisms (Table 1). These organisms possess various degrees of phenotypic or genotypic relatedness to L. pneumophila based in part on DNA homology studies. They have since been classified as representing five species of the genus Legionella. Plasmid elements could not be detected in the Legionella-like strains by the protocol used for plasmid isolation from the Atlanta-1 and -2 strains of L. pneumophila. We therefore developed an isolation procedure which incorporated the central features of several well-established methods.

Table 1. Legionella-like Strains

Legionella pneumophila	Legionella bozemanii
OLDA	WIGA, MI-15
Legionella micdadei	Legionella dumoffii
TATLOCK, HEBA, PPA	TEX-KL, NY-23
Legionella gormanii	
LS-13	

Legionella-like bacteria were cultured on chemically defined medium according to established parameters of growth for L. pneumophila (11). Cells from 100 ml of exponential-phase cultures were harvested by centrifugation and washed once in 10 ml of 10 mM sodium phosphate buffer (pH 7.0). Washed cells were suspended in 3.0 ml of 25% sucrose in 50 mM Tris (pH 8.0), lysozyme (3.0 mg/ml) was added, and the suspension was incubated at 37°C in a shaker-incubator. After 15 to 20 min of incubation, 3.0 ml of 250 mM EDTA (pH 8.0) was added, and the cells were chilled on ice for 5 min. Cell lysis was achieved by the addition of 1.5 ml of 20% sodium dodecyl sulfate followed immediately by incubation in a 55°C water bath for 5 min with gentle agitation. Freshly prepared 3 N NaOH was added drop-wise until the pH was 12.1 to 12.4. The pH was immediately reduced to 8.5 to 9.0 with 2 M Tris (pH 7.0). Denatured chromosomal DNA and cellular debris were precipitated by the addition of 1.5 ml of 20% sodium dodecyl sulfate and 3.0 ml of 5 M NaCl followed by overnight storage at 4°C. The following day, the lysate was centifuged for 30 min at 17,000 x g at 4°C. The precipitate was discarded, and ribonuclease (2 mg/ml in distilled water, heated to 100°C for 5 min) was added to the supernatant to a final concentration of 100 µg/ml and incubated for 30 min at 37°C. Plasmid DNA was precipitated by the addition of 0.05 volume of 3 M sodium acetate and two volumes of cold 95% ethanol and stored at -20°C for at least 4 h. Plasmid DNA was concentrated for 30 min at 17,000 x g, and the resultant pellet was suspended in 100 to 200 µl of Tris-borate buffer. Samples were subjected to electrophoresis in 0.8 and 1% agarose, using Tris-borate running buffer and tracking dye, and stained as previously described by Meyers et al. (7). Samples were electrophoresed at 2 mA for 60 min followed by 50 mA

for 90 to 210 min depending on the degree of band separation desired.

RESULTS

The migration patterns of purified plasmid DNA from the OLDA, WIGA, and TEX-KL isolates are shown in Fig. 1. Molecular weight estimates were determined from the relative migration rates of plasmid bands in agarose gels (Fig. 2). The OLDA strain of L. pneumophila contained a single covalently closed circular plasmid species, pLP3 (Fig. 1D), with an esti- mated molecular weight of 59.8 x 10^6. This was the largest of the five plasmid isolates. The WIGA bacterium contained two plasmid species, pLB1
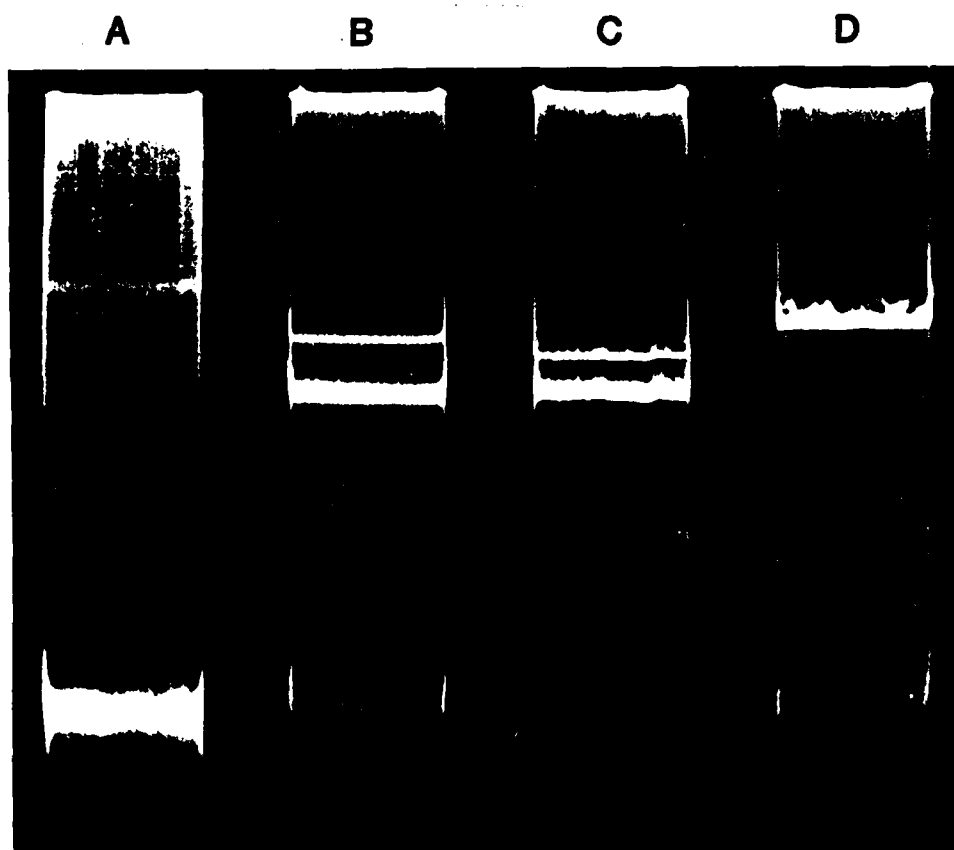

Figure 1. Gel electrophoresis of purified plasmids from Legionella- like bacteria. Purified DNA (30 µl) was mixed with 40 µl of tracking dye. The DNA-dye mixture (30 µl) was applied to agarose well. DNA sam- ples were subjected to electrophoresis in 1% agarose at 2 mA for 60 min followed by 50 mA for 210 min. (A) Pseudomonas aeruginosa, PU21, con- trol; (B) TEX-KL strain; (C) WIGA isolate; (D) OLDA strain of L. pneu- mophila.
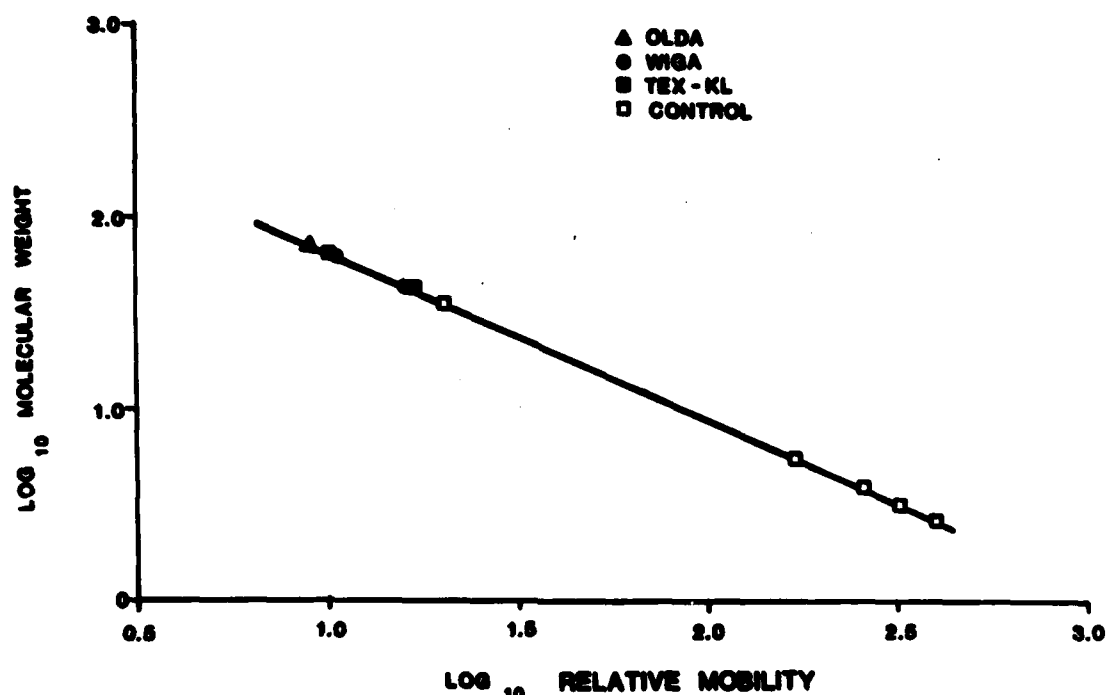
Figure 2. Least-squares regression analysis of plasmid DNA. Plasmid molecular weights were calculated as described by Hansen and Olsen (8). Plasmid DNA from E. coli V517 purified by CsCl-EtBr buoyant density centrifugation was used as a marker strain. This strain contains eight plasmid species. Plasmid molecular weights of marker strain are 35.8 x 10^6, 4.8 x 10^6, 3.7 x 10^6, 3.4 x 10^6, and 2.6 x 10^6. The three smaller plasmid species of the marker strain (2 x 10^6, 1.8 x 10^6, and 1.4 x 10^6) were not retained on the gel under the stated electrophoretic conditions.

(molecular weight, 54.3 x 10^6) and pLB2 (molecular weight, 47.6 x 10^6) (Fig. 1C), the smaller of the two having a double-band appearance. The third intermediate band is believed to be a catenated form of the smaller of the two plasmids (pLB2) in the WIGA isolate and not an open circular form of pLB2 or a third distinct plasmid species. The TEX-KL organism also had two plasmid species, pLK1 and pLK2, with molecular weights of 58.6 x 10^6 and 46.6 x 10^6, respectively (Fig. 1B). The alteration of selected electrophoretic parameters shows that all plasmid isolates are unique entities. Plasmid DNA has also been purified from two additional strains of Legionella-like bacteria (data not shown). LS-13 contains a single plasmid species with a molecular weight of 50-60 x 10^6. The NY-23 strain contains three distinct plasmid elements with molecular weights ranging from 35 to 60 x 10^6. Plasmid DNA was not detected in the HEBA,

TATLOCK, MI-15, or five Pittsburgh pneumonia agent isolates. The failure to isolate extrachromosomal DNA from these organisms may have been due to shortcomings in our technique, and alternate methodologies may eventually establish the presence of plasmid DNA in these organisms. The small 20×10^6-molecular-weight cryptic plasmid of the control P. aeruginosa strain was not observed (Fig. 1A). It is possible that this culture was cured of this smaller plasmid, since extrachromosomal DNA from E. coli in the molecular weight range of 10×10^6 to 20×10^6 was successfully resolved by our protocol (data not shown).

The recovery of plasmid DNA from cells grown in complex medium was very low compared with recovery of plasmid material from cells grown in chemically defined medium. Although these plasmid species do not have high molecular weights, they appear to be highly susceptible to shear forces, which may explain our initial failures in attempting to isolate extrachromosomal DNA by conventional procedures.

DISCUSSION

The isolation of extrachromosomal DNA from members of the Legionella genus is not surprising, considering the ubiquitous nature of plasmid elements. Our results indicate that members of this genus, like other human pathogenic microorganisms, are able to maintain stable plasmid DNA as part of their total genetic complement. Since we have found naturally occurring plasmids in seven strains of Legionella, it seems reasonable that these organisms could support the replication of other plasmids. In recent years, there have been epidemics of typhoid fever in Vietnam and Mexico due to Salmonella typhi having plasmid-mediated resistance to chloramphenicol (9) and the emergence of Haemophilus influenzae resistant to ampicillin (10). Indigenous cryptic plasmids, such as those found in Legionella may acquire transposons which code for drug resistance. The presence of an R-plasmid coding for resistance to erythromycin would severely restrict the present treatment of legionellosis. Although rapid progress has been made in the diagnosis and treatment of this disease, there is still much to be understood concerning the genetics of Legionella: the reason for the difference between the pneumonic and nonpneumonic forms, its precise etiological niche, its nutritional supply, and its phylogenetic relationship to other gram-negative bacteria.

There have been two documented outbreaks of legionellosis in the military population (12, 13). These two epidemics were called "Fort Bragg fever." The etiologic agent was described as rickettsia-like; however, it was never shown conclusively that this organism was responsible for the 1942-43 Fort Bragg epidemics. The organism originally designated TATLOCK, after the individual who isolated the bacterium, has been subsequently classified as a strain of L. micdadei (14). It has been estimated that there are approximately 26,000 undiagnosied cases of legionellosis in the United States each year (2). In view of these morbidity estimates and in consideration of the amount of time which has passed since the organism

was implicated in a military epidemic, there is a significantly high probability that many cases of respiratory disease in military communities have been or are caused by members of the genus Legionella.

The significance of our research with Legionella has not had an immediate impact on the diagnosis and treatment of legionellosis in the military. Our research program was designed to isolate and characterize naturally occurring and genetically engineered plasmids in pathogens of military importance. We successfully established a technological base for this program, in high containment laboratories, using an organism about which virtually nothing was known when we initiated the studies.

The ultimate goal of the program is to contribute to the diagnosis, treatment and prophylaxis of diseases considered to be of military importance. As an extension of our current research program, we have recently demonstrated an association between plasmid DNA and toxin production in Bacillus anthracis. We anticipate that these findings and the application of recombinant DNA technology will contribute to our attempts to develop a more efficacious, long-lasting human anthrax vaccine.

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